RESEARCH PLAN PROPOSAL

MOLECULAR AND BIOCHEMICAL ANALYSIS OF ARSENITE OXIDATION IN BACTERIA ISOLATED FROM ARSENIC CONTAMINATED SOIL

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INTRODUCTION

Arsenic has gained attention of the researchers due its unprecedented release by natural and anthropogenic sources. Arsenic basically occurs in two forms i.e. organic and inorganic forms. Organic arsenic compounds include arsanilic acid, methylarsonic acid, dimethylarsinic acid (cacodylic acid), and arsenobetaine (WHO, 2000), while inorganic forms include arsenate, arsenite, arsenous acids (H_2AsO_3), arsenic acids (H_2AsO_4) etc.

Some inorganic forms are insoluble while others are soluble (Santini *et al*., 2004).Among the Inorganic and Soluble forms which are of main concern are the trivalent Arsenite ($As^{III}O_2^-$) and pentavalent Arsenate ($As^{V}O_4^{3-}$).Arsenite is 100 times more toxic than arsenate (Quemeneur *et al*., 2010), as it enters the cell (bacteria, yeast and mammals) at neutral pH through aquaglycoporins and binds to sulphydryl group of cystein residues of protein and inactivates them. The adverse effects of arsenic include hyperkeratosis (harden skin), restrictive lung disease, peripheral heart disease (black foot disease), cancers etc. (Das *et al.,* 2004). High concentration of inorganic arsenic has also been reported to cause infertility and miscarriages in females (Walton *et al*., 2004).

The biogeochemical cycle of arsenic involves transformation of arsenic species by process of oxidation, reduction and methylation in micro-organisms (Wu *et al*., 2013). The most toxic form of arsenic i.e. Arsenite can be oxidized by bacteria to 100 times less toxic Arsenate which has proved to be a key step in arsenic bioremediation process. These types of chemical modifications by bacteria can help to overcome arsenic toxic effects from environment.

The arsenite oxidation has been reported in bacteria possessing genes encoding enzyme responsible for the catalysis of arsenic transformation reaction. Bacterial resistance to arsenic has been investigated due to the presence of *aox* (arsenite oxidizing) operon possessing structural (*aox A, aox B),* and regulatory genes (*aox R, aox S)* constituting *aox* operon responsible for detoxification process.

Arsenite oxidase is a periplasmic soluble enzyme which catalyzes arsenite oxidation. The Structure of Arsenite oxidase varies in different species. All known aerobic arsenite oxidases exhibit a heterodimeric structure with molybdopterin (Mo-pterin) and Rieske-like subunits (Anderson *et al*., 1992; Ellis *et al*., 2001; Muller *et al*., 2003; Santini and Hoven, 2004; Hoven and Santini, 2004; Silver and Phung, 2005; Kashyap *et al*., 2006). Williams and co- workers in 1986 firstly purified this

enzyme from β- proteobacterium *Alcaligenes faecalis* and worked on its activity and physical properties.

In this study we plan to analyze the isolated arsenite oxidizing bacteria from contaminated soil for its genetic and molecular basis of oxidation.

Review Of literature

1. Arsenic toxicity

Arsenic is the third element in Group V of the periodic table, which is a natural toxic metalloid and is considered as a carcinogenic agent by the World health Organization. The permissible limit for arsenic in drinking water is 10µg/l as recommended by WHO (world health organization) and FAO (Food and Agriculture Organization According to Union). Arsenic contamination in groundwater has become a major problem worldwide. The contamination of potable waters with a high concentration of arsenic has been reported in several countries including Argentina, Bangladesh, Chile, China, India, Japan, Mexico, Mongolia, Nepal, Poland, Taiwan, Vietnam and some parts of United States of America (Anwar *et al*., 2002; Mitra *et al.*, 2002; Pandey *et al*., 2002; Smith *et al*., 2001; Chowdhary *et al.,* 2000). In India, the contamination of arsenic in water above the permissible limit has been reported in Bengal delta plain sediments (Islam *et al*., 2004), middle Ganga plains of Bihar and U.P (Chakraborty *et al.,* 2003) and the Malda district of West Bengal (Rahaman *et al.,* 2013). In a recent study conducted in two districts of Assam (Cachar and Karimganj districts of Barak Valley) has reported that people are drinking arsenic-contaminated water with concentrations ranging maximum 350 µg/l arsenic in groundwater (Shah 2012).

Arsenic is broadly distributed in the environment as it is naturally present in soil, water and air. The contamination occurs due to excess discharge of arsenic from both natural and anthropogenic processes. Natural geogenic contamination occurs due to mining, smelting and combustion of fossil fuels, forest fires, volcanic activity and other natural processes occurring in the soil, ground and

surface water (Valenzuela 2008; Donahoe *et al.*, 2004; Mandal and Suzuki 2002) while the anthropogenic activities include pesticides, wood preservative manufacturing and in mining residues.

The reports on the occurrence of arsenic in ground water resources and the associated health hazards due to its human consumption have been made from various parts of India and world. Some of these health effects caused due to arsenic include cancer of skin, lung, liver, kidney and bladder (Chen *et al.,* 1992; Col *et al.,* 1999), cardiovascular and neurological effects and arsenicosis and skin lesions (Ng *et al*., 2002; Sun 2004; Oremland *et al.,* 2005; Das *et al.*, 2009; Basu *et al.,* 2013).

Arsenic is found in its both organic as well as inorganic form, in which inorganic forms are more toxic (Hopenhayn *et al*., 2006). The primarily stable oxyanionic inorganic forms found in the environment are Arsenite [As (III)] (As (III) as H_3AsO_3, etc.), and Arsenate [As (V)] (including As (V) as $H_2AsO_4^-$, $HAsO_4^{2-}$, etc.) (Ng *et al*., 2002:Jackson *et al.*, 2003; Oremland *et al*., 2005; Branco *et al.,* 2009; Chang *et al*., 2010). Arsenite is 100 times more toxic (Nakamuro and Sayato, 1981, Cullen and Reimer, 1989; Neff, 1997) than arsenate which is poorly soluble in water and, therefore, less bio available (Mandal and Suzuki 2002).

Arsenate ($As^5O^{3-}_4$), is a structural analogue of phosphate, which can enter cells via the phosphate transport system and is toxic because it can interfere with normal phosphorylation processes by replacing phosphate. The production of unstable arsenylated derivatives inhibits phosphorylation processes (Moore, *et al.,* 1988, Coddington, 1986) i.e. in the presence of pentavalent arsenic; Adenosine Di-Phosphate (ADP) forms ADP-arsenate instead of ATP which subsequently hydrolyzes to create a futile cycle within the cell (Anderson *et al.,* 1992).

Arsenite ($As^{III}O^-_2$) is toxic to cells (bacterial, yeast, mammalian) because it bind to essential sulfhydryl groups of proteins and dithiols such as glutaredoxin, which thereby disrupts intracellular oxidation- reduction homeostasis and inhibits pyruvate dehydrogenase. This affinity of arsenite for dithiols can be used by the use of 2, 3-dimercaptopropanol (British antilewisite) as an antidote to arsenite poisoning, as dimercaptopropanol displaces arsenite from proteins by the formation of a stable complex with arsenite (Anderson *et al*., 1992).

In unionized form at neutral pH, arsenite can passively transported across the membrane bilayer, inhibiting cellular glucose uptake, gluconeogenesis, fatty acid oxidation and further production of acetyl CoA. (Marcus 2009).

Bioremediation of Arsenic:-

In recent years, the scientific community has developed many physical, chemical and biological (bioremediation) techniques for the mitigation of arsenic poisoning in drinking water. Bioremediation of heavy or toxic metal contaminated sites has shown to be more efficient alternative than chemical and physical methods as they are cost effective, eco friendly and less toxic. (Valls *et al.,* 2006; Michael *et al.*, 2007).

Review on bioremediation studies with special emphasis on biosorption of different arsenic species by plants and their biomass, agricultural and industry wastes, as well as the biomass of some fungi species has also been done. Among the bioremediation technique, phytoremediation is one of the popular methods which use plants to remove arsenic from the environment; it also helps in transforming arsenic to less toxic form. Differences in arsenic uptake by different plant species varies with factors such as root surface area, root exudates, and rate of evapotranspiration (Dabrowska *et al* ., 2012 ; Tsai *et al.,* 2012) .

Some other approaches of amelioration are also promising which include the use of biofilm and siderophore. However, microbially enhanced phytoextraction with special reference to establishments of mycorrhizal fungi in rhizosphere of arsenic hyperaccumulating Chinese Brake Fern (*Pteris vittata* L.) hold promise (Purakayastha *et al*., 2011).

Microbial approaches are also promising as the use of engineered microbes as selective biosorbents is an attractive green cure technology for the low cost and efficient removal of arsenic from soil (Purakayastha *et al.,* 2011).

Microbes have been reported to develop arsenic resistance by arsenite oxidation to arsenate and thereby reducing its toxicity by 100 times. Thus As(III)-oxidizing bacteria can contribute to natural attenuation of As pollution by decreasing its bioavailability and can help remediate As from mine wastewaters through bioprocessing (Battaglia-Brunet *et al*., 2002; Battaglia-Brunet *et al.,* 2006). A previous study showed that chemical reducing condition was a key factor affecting the dissolution of arsenic from sediments to groundwater and the authors pointed out that the transformation of arsenic may mainly be due to microbial metabolisms (Guo *et al*., 2003). Developing environment friendly and efficient technology to remove arsenic from soil is essential in affected areas (Alaerts *et al*., 2004).

Arsenite oxidation has been observed in mammals, including humans (Vahter *et al*., 1983), and in bacteria (Green *et al.,* 1918; Osborne *et al.,* 1976) .Various heterotrophic bacteria have been reported which generally oxidize As (III) for detoxification, and they do not gain energy through this arsenite

oxidation process (Anderson *et al.,* 1992; Martin and Pederson, 2004; Cai *et al.,* 2009). Similarly, various reports have been available on arsenite oxidation by Chemoautrophic or Chemolithotrophic bacteria, which use As (III) as an electron donor, oxygen as the terminal electron acceptor, and carbon dioxide as a sole carbon source (Santini *et al*., 2000; Bernhardt and Santini, 2006). Arsenic transformation in Bacteria is mediated by different strategies like arsenite oxidation, cytoplasmic arsenate reduction, respiratory arsenate reduction, and arsenite methylation mechanism (Mukhopadhya 2002; Oremland *et al.,* 2002; Silver and Phung 2005; Bhattacharya *et al.,* 2007; Purakayastha *et al.,* 2011).

Arsenite oxidation is induced in the presence of arsenite as revealed in some strains of the soil bacterium *Alcaligenes faecalis* (Osborne *et al.,* 1976, Phillips *et al.,* 1976). As (III) oxidation has been identified in various bacteria including *Pseudomonas* (Turner 1954). *Alcaligenes , Thiomonas ,Herminiimonas , Agrobacterium* and *Thermus*(Cai *et al*., 2009) Thus, Microbial activity may play a vital role in global As cycling by converting arsenite to less mobile and less toxic arsenate species (Inskeep *et al*., 2002).

Genes Responsible for arsenite oxidation:-

The potential detoxification processes of arsenic by microorganisms may be related to arsenic resistance gene (*ars*), arsenic respiratory reduction genes (*arr*) or arsenite oxidation genes (*aox* etc.) (Ji and Silver 1992b; Kashyap *et al*., 2006).Genes encoding arsenite oxidizing enzyme have been described in the bacteria like: *Alcaligenes faecalis, Cenibacterium* ars*enoxidans, Thermus sp., Thermus thermophilus and Agrobacterium tumefaciens* (Silver and Phung 2005; Kashyap *et al*., 2006). Nine bacterial strains, identified to be *Pseudomonas* were isolated from Camarones river sediments, located in Northern Chile, a river which is highly contaminated with arsenic concentrations up to 1, 100 µg/L. The arsenite oxidase genes were identified in eight out of nine isolates they had their potential application in biological treatment of effluents contaminated with arsenic (Valenzuela *et al.,* 2009).

Genes encoding As(III) oxidases (*aox*) are organized in an operonic structure which have also been identified and sequenced in several organisms, showing a common genetic organization, *aoxA-aoxB*, that encodes the small and large subunits, respectively (Muller *et al.,* 2003; Santini *et al.,* 2004; Alvarez-Martinez *et al.,* 2007; Muller and Medigue *et al.,* 2007). These *aox* operons usually contain additional genes, e.g., *cytC*, which encodes cytochrome *c*, and *moeA*, which encodes an enzyme involved in molybdenum cofactor biosynthesis (Silver and Phung., 2005). *Pseudomonas stutzeri*

strain GIST-BDan2 (EF429003) was reported to show arsenic detoxification by its oxidation, and it was revealed that the presence of *aox*B and *aox*R gene could play an important role in transformation of arsenite to arsenate. The researchers concluded that the strain completely oxidizes 1 mM of As(III) to As(V) within 25–30 h. (Chang *et al.,* 2010).

In recent study on *Agrobacterium tumefaciens,* suggest the presence of a complex transcriptional regulation involving As(III) sensing, two-component signal transduction by an AoxS sensor kinase and an AoxR regulator and quorum sensing (Kashyap *et al*., 2006).

It has been reported that arsenite-oxidizing bacteria such as *β-proteobacterial* strain ULPAs1

(having *aox*A and *aox*B genes) and *Agrobacterium tumefaciens* (having a signal transduction system; *aox*S-*aox*R-*aox*A-*aox*B-*cytc*2) were isolated from the arsenic-contaminated water and soil by Muller *et al*., 2003. The studies suggested that arsenic-oxidases played critical roles in the arsenic detoxification processes by the *aox*S-*aox*R- *aox*A-*aox*B-*cytc*2 systems, which explain the regulatory control of *aox*AB.

Enzyme:-Arsenate oxidase

As reported by Anderson *et al.,* 1992, arsenite oxidation is catalyzed by arsenite oxidase which is a

periplasmic soluble enzyme which was firstly purified in 1992 from *β- Proteobacterium Alcaligenes*

faecalis. Arsenite oxidases was also purified from a chemolithoautotrophic arsenite oxidizer NT-

26,which is a member of *α-Proteobacteria*, and *Hydrogenophaga* sp. str. NT-14 has been studied in

detail where the enzyme is located in the periplasmic space (Santini *et al*., 2000; Vander *et al*., 2004). Among the heterotrophic arsenite oxidizers, *Alcaligenes faecalis* and ULPAs1, members of

β- Proteobacteria, the enzyme anchored to the periplasmic face of the inner membrane (Anderson *et al*., 1992; Muller *et al*., 2003). Homology between the sequences at the amino acid level varied from 72% to less than 52% among the purified enzymes derived from these three organisms. Owing to low homology in the sequence and differences in the localization of the enzyme within the cell, enzyme arsenite oxidase may have evolved separately in the two types of metabolically different organisms—autotrophs and heterotrophs.

All known aerobic arsenite oxidases exhibit a heterodimeric structure with molybdopterin (Mo-pterin) and Rieske-like subunits (Anderson et al., 1992; Ellis et al., 2001; Muller et al., 2003; Santini and Hoven, 2004; Hoven and Santini, 2004; Silver and Phung, 2005; Kashyap et al., 2006).

3D structure of this enzyme has also been reported (Ellis et al., 2001) which showed that this arsenite oxidase is a heterodimeric enzyme with small and large subunits. These two subunits are encoded by the genes aoxA/aroB/asoB (small Fe-S Rieske subunit) and aoxB/aroA/asoA (large Mo-pterin subunit) respectively (Mukhopadhaya et al., 2002 and Santini et al., 2004). Recently aoxB-like sequences have been widely found in different arsenic contaminated soil and water systems (Inskeep et al., 2007). Researchers have reported arsenite oxidase to possess small and large subunits consisting of approximately 825 and 134 amino acid residues respectively. The larger subunit consists of Mo site having two pterin cofactors bound with Mo atom and a [3Fe-4S] cluster. The smaller subunit possesses Rieske-type [2Fe-2S] site (Carrell et al., 1997; Ellis et al., 2001) found in Rieske/cytb complexes (formerly called cytochrome bc or cytochrome bc–type complexes (Schu¨ tz et al., 2000), in dioxygenases (Schmidt and Shaw 2001) and phytoene desaturases. All enzymes in this family are involved in electron transport whereby the Mo centre serves to cycle electrons via the MoIV and MoVI valence states, and appear to have a common ancestor present prior to the divergence of Bacteria and Archaea (Ewan *et al*., 2002; Lebrun *et al*., 2003). The large subunit (AroA ~90 kDa) of the arsenite oxidase is the first example of a new subgroup of the dimethylsulfoxide (DMSO) reductase family of molybdoenzymes (Ellis *et al*., 2001). Interestingly, the large subunit (ArrA) of the dissimilatory arsenate reductase also contains Mo but is not related to the known AroA proteins. According to inskeep *et al* 2007 all the three known arsenite oxidase homologues are AroA, AsoA, AoxB.

Arsenite oxidase genes have been described in the following bacteria: *Alcaligenes faecalis, Cenibacterium arsenoxidans, Thermus sp., Thermus thermophilus and Agrobacterium tumefaciens* (Silver and Phung 2005; Kashyap *et al*., 2006). It is actually composed of two sub-units encoded by two genes included in an operon (Silver and Phung 2005). Other arsenite-oxidases and genes involved in As (III) oxidation have now been characterised in several micro-organisms (Lebrun *et al*., 2003; Muller *et al*., 2003; Silver and Phung 2005).

SIGNIFICANCE OF WORK

Arsenic contamination in soil and groundwater systems has gained attention of researchers over past decades. Several mechanisms of arsenic transformation have been proposed like oxidation, reduction and methylation. The oxidation of most toxic form arsenic (i.e. conversion of arsenite to arsenate) is a key step of detoxification mechanism by microbes. Thus, the exploration of the molecular mechanism in arsenic oxidizing bacteria from contaminated soil is highly significant for the arsenic bioremediation process.

In this research proposal, the molecular approach for arsenite oxidation in the isolated bacterial strain will be analysed by amplifying the genes responsible for biotransformation of arsenite to arsenate. Further the partial purification of enzyme and monitoring its enzyme activity will also be done to confer its potential role in bioremediation.

OBJECTIVES OF RESEARCH

1. Characterization of arsenite oxidizing Bacterial isolates

2. Optimization of growth conditions

3. Partial purification of the enzyme responsible for arsenite oxidation

METHODOLOGY

i. Isolation of Arsenite oxidizing bacteria:-

Using Dilution plating technique Arsenite oxidizing bacteria will be isolated from arsenic contaminated soil taken at different depths. Arsenite resistant bacteria will be cultured in

defined medium supplemented with 1000ppm (7.69mM or 1g/L) of sodium arsenite and incubated at 37°C for 24-48 h at 120 rpm. Repeated spreading and streaking will help to isolate pure colonies. The strains will be preserved in 15 % v/v of glycerol.

ii. **Determination Minimum Inhibitory Concentration (MIC) of Arsenite hypertolerant bacteria:-**

Isolated pure colonies will be inoculated in defined medium supplemented with increasing doses of sodium arsenite at different temperature and durations of time. The lowest concentration at which the growth ceases will be considered its MIC (Courvalin *et al.*, 1985; Muller *et al.,* 2003), which will be determined by optical density of the culture at 600nm (UV-Vis Spectrophotometer) on the basis of negligible growth.

iii. **Optimization of growth conditions: -**

The arsenite oxidizing bacterial growth conditions will be standardized for Media composition (Mohapatra *et al*., 2005), pH, Temperature (Rehman *et al*., 2010).

iv. **Analysis of oxidizing ability:-**The oxidizing ability of isolated bacteria showing highest MIC will be checked by-

a) **Preliminary Test:** The analysis for arsenite oxidizing ability will be conducted by Silver Nitrate Screening by flooding the solution of silver nitrate onto agar plates (Lett *et al.*, 2001).

b) **Confirmatory Test:** Determination of oxidation rate of arsenite to arsenate will be done by Molybdene Blue Spectrophotometric Method (Zhou, 1990; Lenoble *et al.,* 2003; Cai *et al.,* 2009b) followed by the confirmation of the same with anion Exchange Chromatography followed by Atomic Absorption Spectroscopy (Samanta *et al.,* 1999; Le *et al.,* 2000; Alauddin *et al.,* 2003).

v. **16S rDNA sequencing and biochemical characterization of the isolated strains:** The oxidizing strains will be identified by sequencing of the amplified 16S rDNA fragment. The sequencing results will be supported by biochemical characterization using Bergey's Manual of Determinative Bacteriology, 1994.

vi. **Molecular analysis of oxidizing ability:** The genes responsible for arsenic detoxification potential of the bacterial strains, like *aox* A, *aox* B, *aox* R and *aox* S forming an operon system will be investigated. This will be done by PCR amplification of *aox* genes using specific primers (Quemeneur *et al.*, 2008; Chang *et al*., 2009). The amplification of various

aox genes will confirm the presence of arsenite oxidase (enzyme responsible for the oxidation process of arsenite to arsenate).

vii. Partial Purification of Enzyme:

a) Cell free extract preparation:-

The extract of cultural bacterial isolate cells will be prepared by Sonication and centrifugation. (Anderson *et al*., 1992; Prasad *et al*., 2009)

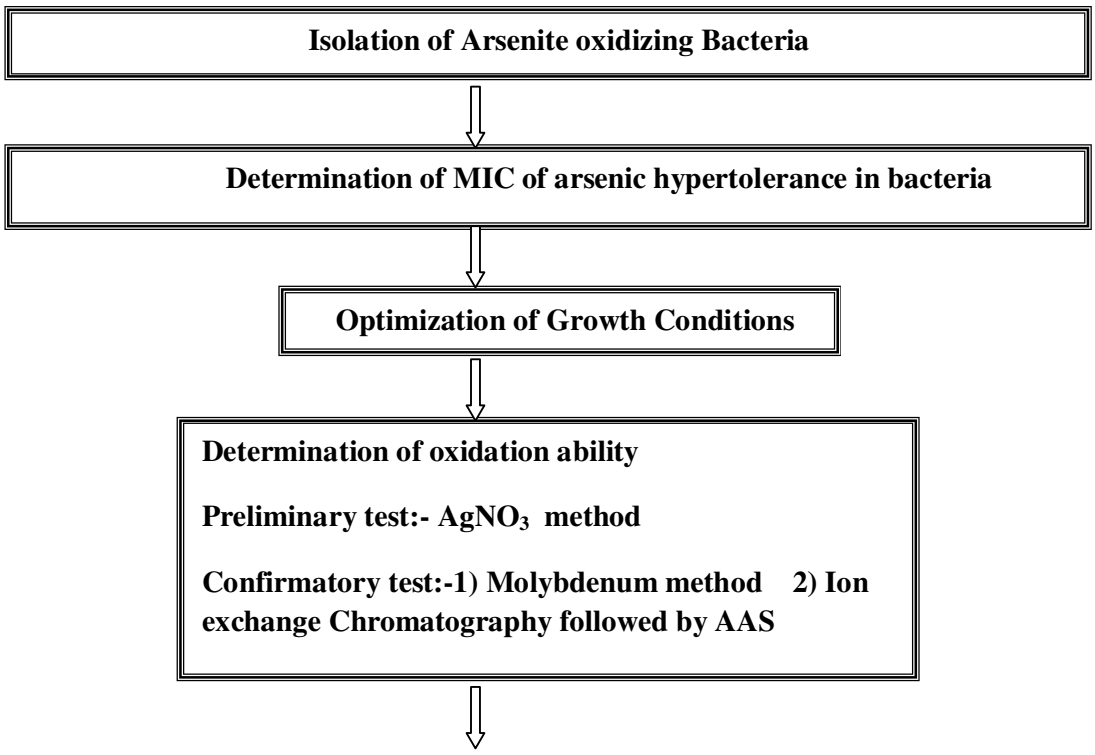
b) Purification of enzyme responsible for arsenite oxidation:-

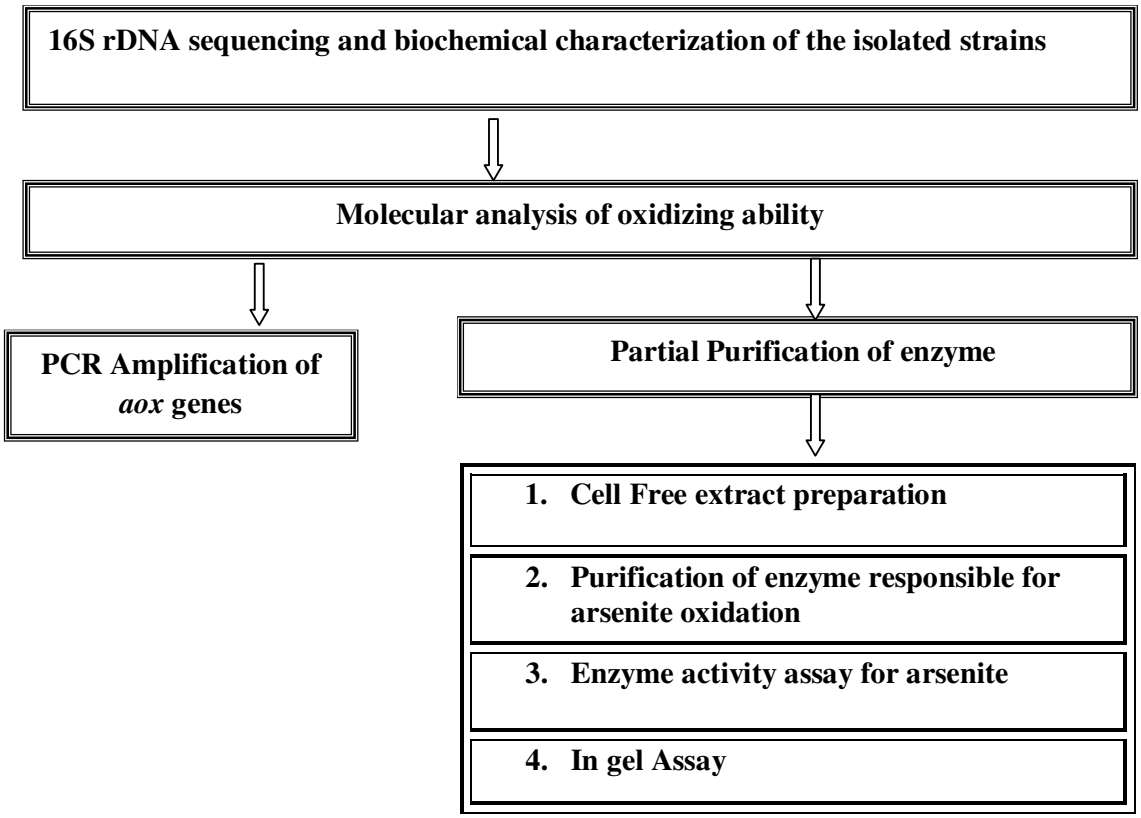
Protein will be precipitated with different concentration of ammonium sulphate (10% to70% saturation) and purified with Gel Chromatography, Dialysis and ion exchange chromatography techniques Bradford method (Prasad *et al*., 2009; Santini *et al*., 2004) and Lowry method will be used for estimating protein content. (Coughlan *et al*., 1969; Anderson *et al*., 1992; Michael *et al*., 2007; Rehmann *et al*., 2010)

c) Enzyme activity assay for arsenite oxidation:-

The activity assay of fraction purified will be performed with biochemical estimations like an artificial electron acceptor i.e DCIP (2,6-dichlorophenol indophenol) and MES (morpholino ehthelenediol sulphonic acid), where in reduction of the same will be monitored at 600nm in the presence of sodium arsenate in MES buffer which will depict that the enzyme is responsible for initiating oxidation (Philips 1976; Anderson *et al*., 1992; Muller *et al*., 2003; Mohapatra *et al*., 2006; Prasad *et al*., 2009; Richey *et al*., 2009). The Purified protein will also be studied by In gel Assay (Poly Acrylamide Gel Electrophoresis), stained with DCIP. Gel will be rinsed with MES followed by flooding of sodium arsenate until the achromatic zone develops in the gel depicting the presence of enzyme (Prasad *et al*., 2009).

WORK PLAN


Isolation of Arsenite oxidizing Bacteria
Determination of MIC of arsenic hypertolerance in bacteria
Optimization of Growth Conditions
Determination of oxidation ability
Preliminary test:- $AgNO_3$ method
Confirmatory test:-1) Molybdenum method 2) Ion exchange Chromatography followed by AAS


16S rDNA sequencing and biochemical characterization of the isolated strains
Molecular analysis of oxidizing ability
PCR Amplification of aox genes
Partial Purification of enzyme
1. Cell Free extract preparation
2. Purification of enzyme responsible for arsenite oxidation
3. Enzyme activity assay for arsenite
4. In gel Assay

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